Original Article

NCOA2-induced secretion of leptin leads to fetal growth restriction via the NF-κB signaling pathway

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Background: Fetal growth restriction (FGR) is one of the most common fetal complications during pregnancy in the obstetrics department, with poor therapeutic efficacy. The local inflammatory response of the placenta has gradually become known as the main mechanism for the occurrence and development of FGR. The aim of this study was to improve the knowledge of placental inflammatory response mechanisms in regulating gene expression.

Methods: The differentially expressed genes (DEGs) in FGR patients were analyzed through bioinformatics analysis. The expression of gene level was detected by immunohistochemistry (IHC) staining, quantitative polymerase chain reaction (qPCR), or enzyme-linked immunosorbent assay (ELISA). The proliferation, migration, and apoptosis of HTR-8/SVneo trophoblast cells stimulated with lipopolysaccharide (LPS) was performed by Cell Counting Kit-8 (CCK-8) assay, clone formation assay, Transwell assay, and flow cytometry. The mechanisms of gene expression in regulating placental inflammatory response were elucidated by western blotting.

Results: Nuclear receptor coactivator 2 (*NCOA2*) was identified as a very critical gene in the progression of FGR by bioinformatics analysis and the expression of *NCOA2* was shown to be down-regulated in FGR patients. Overexpression of *NCOA2* promoted the proliferation, migration, and inhibited apoptosis and pro-inflammatory cytokines secretion in HTR-8/SVneo trophoblast cells stimulated with LPS via the nuclear factor (NF)-κB pathway. In addition, leptin was increased in both tissue and peripheral blood samples of FGR patients, and overexpression of *NCOA2* inhibited the secretion of leptin in HTR-8/SVneo trophoblast cells stimulated with LPS.

Conclusions: All these findings suggest that *NCOA2*-induced secretion of leptin leads to FGR progression via the NF-κB pathway and provides a clinical therapeutic target in FGR and a potent marker for the identification of FGR.

Keywords: Fetal growth restriction (FGR); inflammatory response; nuclear receptor coactivator 2 (*NCOA2*); leptin; NF-κB pathway

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Introduction

Due to maternal and fetal pathological factors affecting the placenta, fetal growth may not reach its due genetic potential, defined as fetal growth restriction (FGR) or intrauterine growth restriction (IUGR) (1). FGR is the second leading cause of perinatal death worldwide with an incidence rate in China of 6.39% (2). FGR is an important cause of perinatal morbidity and mortality, and may also lead to long-term adverse outcomes, including childhood cognitive impairment and increased risk of adult-onset diseases (such as obesity, type 2 diabetes, cardiovascular disease, stroke, etc.) (3). The placenta is an important organ for maternal-fetal material exchange and dysfunction of placenta is the leading cause of FGR (4). Thus, an understanding of the crucial role and the molecular mechanism of placental dysfunction may offer insight into early therapeutic strategies of FGR.

The incidence of FGR is often affected by many factors, which can be generally summarized as maternal, fetal and placental inflammation. All these factors often do not exist in a single form, among which placental factors are the most important cause. The inflammatory reaction of placenta leads to the increase of apoptosis of trophoblast cells, the decrease of proliferation and motor ability, and the decrease of the formation of blood differentiation, which leads to the growth and development restriction of fetus (5,6). FGR is considered a disease closely related to inflammation. It has been reported that multiple factors lead to maternal inflammation, including infectious or non-infectious inflammation, which are closely related to the occurrence of FGR (7). Studies have found that the levels of pro-inflammatory cytokines, such as interleukin-8 (IL-8), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), in the peripheral blood of patients with FGR caused by placental dysfunction are increased (8,9). These results suggest that inflammatory factors may induce increased apoptosis of trophoblast cells, placental angiogenesis, and dysfunction, which may affect nutrient exchange between mother and fetus and further lead to the occurrence of FGR. Nuclear factor-κB (NF-κB) is a family of inducible transcription factors that regulate a large number of genes involved in different processes of immune and inflammatory responses (10). Inflammatory pathways through placental NF-κB activation and production and secretion of pro-inflammatory cytokines (IL-6 and TNF-α) are associated with fetal systemic inflammation, which increases placental dysfunction (11).

Leptin is a kind of non-glycosylated protein mainly produced by fat cells, which has dual functions of hormone and cytokine. When acting as a cytokine, it has obvious proinflammatory effect. In obese patients, for example, elevated leptin levels promote low-grade inflammatory states, making this population more likely to develop cardiovascular disease, type 2 diabetes or degenerative diseases, as well as autoimmune diseases; In people with low leptin levels, such as malnourished people, infection risk increased and resistance decreased, which may be related to the activation of Treg cells that suppress immune response. As a hormone, it influences various endocrine functions and bone metabolism, in addition to regulating key functions of energy homeostasis through mechanisms including thermoregulation. In obstetric diseases, Leptin is considered to be a molecular marker for the occurrence and development of Pre-Eclampsia (PE) and FGR, and the increase of its blood level is positively correlated with the occurrence and severity of FGR and PE (12,13). In Schoots *et al.*, Leptin can be used as a molecular marker to predict the occurrence of these two common gynecological diseases in FGR and PE (14). At the same time, Mise *et al.* have also confirmed that maternal leptin level can reflect the deterioration of fetal somatic restriction (15).

In the present study, we carried out a microarray analysis of differentially expressed genes (DEGs) between the normal and FGR patients and revealed that nuclear receptor coactivator 2 (*NCOA2*) is a very critical gene in the progression of FGR. The *NCOA2* gene is situated on chromosome 8q13.3, a member of the steroid receptor coactivator family, assisting the function of nuclear hormone

Highlight box

Key findings

- NCOA2-induced secretion of leptin leads to FGR progression via the NF-κB pathway and provides a clinical therapeutic target in FGR.

What is known and what is new?

- The local inflammatory response of the placenta has gradually become recognized as the main mechanism for the occurrence and development of FGR.
- This manuscript adds to the knowledge of placental inflammatory response mechanisms in regulating gene expression and function of NCOA2.

What is the implication, and what should change now?

- The function and mechanism of NCOA2 provide a potent application in the treatment of FGR.

receptors (16). As a PPARr coactivator, it plays an important role in lipid metabolism and energy balance (17). *NCOA2* plays a crucial role in the occurrence, development, and metastasis of many malignant tumors such as mesenchymal chondrosarcoma (18), prostate cancer (19), gastric cancer (20), leukemias (21), and spindle-cell rhabdomyosarcoma (22). Li *et al.* had shown that the messenger RNA (mRNA) and protein levels of *NCOA2* were dose-dependently increased by treatment with lipopolysaccharide (LPS), a well-known inflammation trigger (23), indicating that *NCOA2* may participate in the regulation of inflammation. However, the expression of *NCOA2* in FGR placentas and the role of *NCOA2* in FGR through the inflammatory pathway have not been reported.

In this study, we aimed to investigate the potential role and regulatory functions and mechanisms of *NCOA2* in HTR-8/SVneo trophoblast cells. Our results demonstrated that pro-inflammatory cytokines (IL-1β and IL-6) were increased in FGR patients, indicating that inflammation participates in the progress of FGR. The expression of *NCOA2* was down-regulated in FGR patients. Overexpression of *NCOA2* promoted the proliferation and migration, and inhibited the apoptosis and secretion of pro-inflammatory cytokines in HTR-8/SVneo trophoblast cells stimulated with LPS via the NF-κB pathway, and provided a clinical therapeutic target in FGR. In addition, leptin was shown to be increased in both tissue and peripheral blood samples of FGR patients. Overexpression of *NCOA2* inhibited the secretion of leptin in HTR-8/SVneo trophoblast cells stimulated with LPS, which providing a potent marker for the identification of FGR. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6444/rc).

Methods

Data extraction

The microarray datasets (GSE24129 and GSE12216) were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). These datasets included 16 samples from normotensive pregnancies with FGR and 16 from normotensive control pregnancies. These datasets were produced by an Affymetrix Human Gene 1.0 ST Array platform (Affymetrix, Santa Clara, CA, USA). All microarrays from the 2 datasets were downloaded and normalized and $\log_2$ transformed.

DEGs analysis

To identify the DEGs, these datasets were analyzed with the R software "limma" package. Genes with $|\log_2$ fold change (FC)$|$ >1.0 and false discovery rate (FDR) value <0.05 and P<0.05 were defined as DEGs and the volcano map of DEGs was created with the R software "ggplot2" package (R Foundation for Statistical Computing, Vienna, Austria). The overlap/intersection part of the up-regulated and down-regulated DEGs between the 2 GSE datasets was analyzed through the Venn Diagram online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Functional enrichment analysis

The co-expressed differential genes were imported into the ClueGO database of Cytoscape 3.9.1 software (https://cytoscape.org/) for Gene Ontology (GO) enrichment analysis (P<0.05) and visualized by GraphPad Prism 8.0.2 (GraphPad Software, LA Jolla, CA, USA). The significant enrichment screening criteria for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs was explored and visualized using the ggplot2 package of R software.

Protein-protein interaction (PPI) networks

The interaction network between coexpressed DEGs was constructed by the Search Tool for the Identification of Interacting Genes/Proteins (STRING) database (https://string-db.org/) and visualized by Cytoscape (version 3.9.1).

In addition, the "Degree" algorithm in cytoHubba plug-in was used to calculate and analyze the network characteristics of each node.

Human placental tissue collection

First-trimester placental tissues with FGR (36 to 40 weeks of gestation, the test group) or term placental tissues (>37 weeks gestation, the control group) from healthy women were collected from legal pregnancy terminations in the Department of Obstetrics and Gynecology, the First Affiliated Hospital of Nanjing Medical University from December 2018 to October 2019. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). There were no complications in the 2 groups and all experimental procedures were approved by the Research Ethics Committee of the First Affiliated Hospital

of Nanjing Medical University (No. 2020-SR-434). All participants signed a written informed consent.

Cell culture

Human chorionic trophoblast cell line (HTR-8/SVneo cell) was maintained in Roswell Park Memorial Institute (RPMI) 1640 with 5% fetal bovine serum (FBS), 1% penicillin and streptomycin at 37 ℃ and 5% CO_2. NCOA2 small interference RNA (si-NCOA2: 5'-GCACTCTTGTTGCTGCACAAA-3' and negative control (si-NC: 5'-UUCUCCGAACGUGUCACGUTT-3') were synthesized by GenePharma (Shanghai, China). The siRNAs (50 nM) were transfected into HTR-8/SVneo cells using Lipofectamine™ 3000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Cell Counting Kit-8 (CCK-8) assay

CCK-8 analyses were performed according to the manufacturer's instructions. Briefly, cells were harvested in different time points and incubated for 1 hour at 37 ℃ with 10 μL CCK-8 regent (Dojindo, Kumamoto, Japan). The optical density (OD) value was determined at 490 nm by a Bio-Rad microplate reader (Bio-Rad, Hercules, CA, USA).

Colony formation assays

For the colony formation assay, HTR-8/SVneo cells were seeded in triplicate into 6-well plates for 14 days with normal medium. Then, the cells were fixed with 4% paraformaldehyde and stained with crystal violet for 10 minutes, and the colony numbers were determined using a light microscope on 6 randomly selected areas.

Transwell assay

For cell migration analysis, cells were placed in a modified Boyden chamber with 8 μm-pore polycarbonate filter (Corning, New York, NY, USA) in Dulbecco's modified Eagle medium (DMEM) containing 0.1% FBS and the lower chamber was filled with 10% FBS DMEM media. After 24 hours, the cells on the other side of membrane were fixed with 4% formaldehyde solution and stained with 0.5% crystal violet and the number of migrated cells were counted in 5 randomly selected fields by using a microscope (Olympus, Tokyo, Japan).

Human IL-1β, IL-6, and leptin enzyme-linked immunosorbent assay (ELISA)

The level of IL-1β, IL-6, and leptin in peripheral blood obtained from either FGR pregnant women or normal women was determined by ELISA (Elabscience, Wuhan, China). Peripheral blood was centrifuged at 3,000 rpm for 20 minutes and supernatants were harvested and the IL-1β, IL-6, and leptin levels were determined according to the manufacturer's instructions.

Immunohistochemistry (IHC)

IHC was performed according to standard protocols. Briefly, paraffin sections were washed multiple times with xylene to solubilize and remove the paraffin and then rehydrated through graded concentrations of ethanol in water, followed with antigen retrieval by Tris/ethylenediamine tetraacetic acid (EDTA) (pH 8.0) buffer. The sections were then treated with 3% hydrogen peroxide for 10 minutes followed with 5% bovine serum albumin (BSA) for 1 hour at room temperature and then the sections were incubated with a primary antibody overnight at 4 ℃. After incubation with secondary antibodies for 1 hour at room temperature, 3,3'-diaminobenzidine (DAB) was used as the chromogen. All sections were counterstained with hematoxylin and observed the color of the antibody staining in the tissue sections under a light microscopy.

Real-time quantitative polymerase chain reaction (RT-qPCR)

RNA was isolated from HTR-8/SVneo cells using TRIZOL regent and reverse transcribed into complementary DNAs (cDNAs) using a reverse transcription kit (Promega, Madison, WI, USA) according to the manufacturer's instruction, followed by RT-PCR with SYBR Premix Ex Taq using the Bio-Rad CFX96 system. Expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to β-actin. The primer sequences used in PCR were as follows:

NCOA2, F 5'-TCGCCTGGAAGCAGAAAGTT-3', R 5'-GGGGCGTCCATTGGATGTAT-3';

Leptin, F 5'-TGCCTTCCAGAAACGTGATCC-3', R, 5'-CTCTGTGGAGTAGCCTGAAGC-3';

β-actin, F 5'-CTCCATCCTGGCCTCGCTGT-3', R 5'

-CTCCTTAATGTCACGCACGAT-3'.

Western blot assay

Total proteins from HTR-8/SVneo cells were extracted by radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing protease inhibitor (Roche, Indianapolis, IN, USA). Then, almost 30 μg protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transfected onto polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA, USA). The membrane was blocked with 5% BSA and then incubated with the primary antibody and secondary antibody. The visualization of signal blots was achieved by Millipore ECL kit and Bio-Rad ChemiDoc Touch. All antibodies were purchased from HUABIO (Hangzhuo, China) and listed as below: NCOA2 (1:1,000), leptin (1:1,000), IκBα (1:1,000), NF-κB/p65 (1:1,000), p-NF-κB/p65 (1:1,000), GAPDH (1:2,000), and Goat Anti-Mouse or Rabbit IgG (1:50,000).

Statistical analysis

All the experiments were performed in triplicate and the statistical analysis was applied by GraphPad Prism 8 software. Data are presented as mean ± standard deviation (SD). Student's *t*-test or 2-way analysis of variance (ANOVA) was used to analyze the comparisons between 2 or more groups. A P value <0.05 was considered a significant difference.

Results

DEGs identification and functional enrichment analysis

First, in order to identify the DEGs between FGR patients and normal controls, the microarray data of FGR patients was downloaded from the GEO databases and analyzed by the R software. The volcano plot showed the down-regulated genes and up-regulated genes in FGR patients from GSE24129 (*Figure 1A*) and GSE12216 (*Figure 1B*). A total of 4,012 genes (1,339 up-regulated genes and 1,170 up-regulated genes in GSE24129 and 1,138 up-regulated genes and 365 down-regulated genes in GSE12216) were identified as DEGs between 16 FGR patients and 16 normal persons (*Figure 1A*,*1B*).

Co-expression analysis was performed to identify the co-expressed DEGs. According to Venn analysis, 58 co-expressed up-regulated DEGs (*Figure 1C*) and 18 co-expressed down-regulated DEGs (*Figure 1D*) were identified between GSE24129 and GSE12216 and are presented in the heatmap of *Figure 1E*. Then, the KEGG functional annotations of these up- and down-regulated DEGs were applied and the top 10 pathways with P<0.05 were selected. Enriched processes of these DEGs were significantly associated with pathogenic *Escherichia coli* infection, cholesterol metabolism, and cell adhesion molecules (*Figure 1F*).

PPI networks of 58 up- and 18 down-regulated co-expressed genes were constructed using the STRING database. In the PPI network of DEGs, there were 14 nodes and 14 edges, with an average node degree of 4.2. In addition, the Degree (Deg) algorithm in cytoHubba plug-in was used to analyze the network characteristics of each node in PPI network (*Figure 1G*). The genes *LEP*, *C1QA*, and *CLDN5* had the top 3 scores and were considered the key hub genes. Furthermore, the relationship between *NCOA2* and *LEP* has not been reported, and the relationship between *NCOA2* and FGR has not been studied. Therefore, studying the function and effect of *NCOA2* in FGR can provide further theoretical basis for revealing the pathogenesis of FGR.

Validation of the NCOA2 expression and leptin secretion in FGR tissues

We validated the expression of *NCOA2* and leptin in placental samples from 10 FGR patients and 10 normal controls. The IHC staining (*Figure 2A*) and quantitative (q) RT-PCR assay (*Figure 2B*) showed that the expression of *NCOA2* was down-regulated and leptin was up-regulated in FGR patients compared with the normal controls, which was consistent with the microarray data presented in *Figure 1E*. In addition, the secretion of leptin was also significantly increased in the serum of FGR patients (*Figure 2C*), which indicated that the level of leptin in serum could be used to predict FGR in pregnant women. Inflammatory cytokines have been reported to be associated with FGR. Therefore, we decided to measure the levels of proinflammatory cytokines IL-1β and IL-6 in the FGR cases and normal controls by ELISA, which revealed that the expression of IL-1β and IL-6 was significantly increased in FGR patients (*Figure 2C*). These results demonstrated the expression levels of *NCOA2* and leptin in FGR tissues and suggested that an inflammatory reaction is involved in this process.

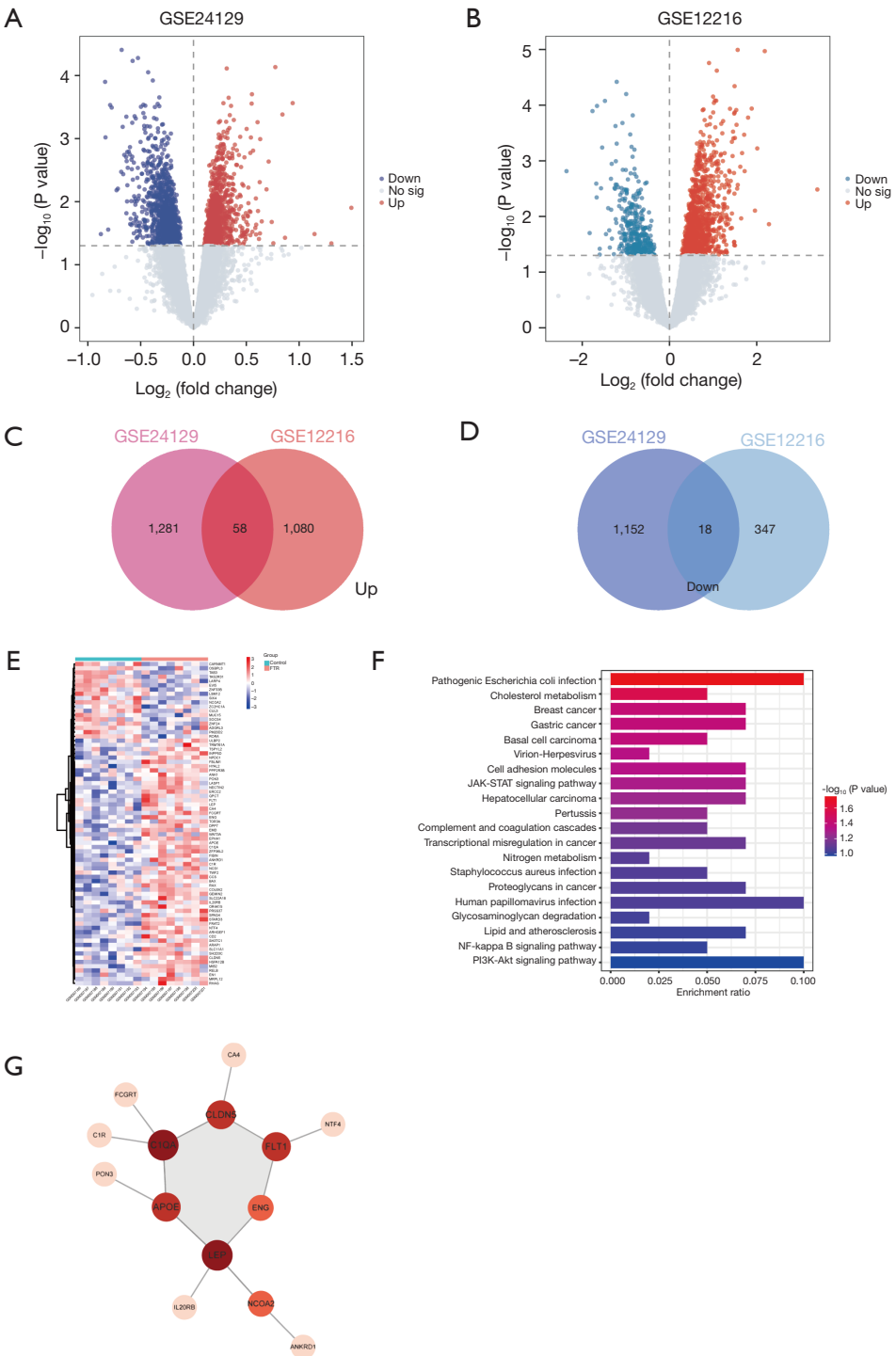


Figure 1 DEGs identification and functional enrichment analysis. (A) The Volcano Plot showed the DEGs from GSE24129. (B) The Volcano Plot showed the DEGs from GSE12216. (C) The Venn analysis of co-expressed up-regulated DEGs between the GSE24129 and GSE12216. (D) The Venn analysis of co-expressed down-regulated DEGs between the GSE24129 and GSE12216. (E) The heatmap of these co-expressed DEGs between the GSE24129 and GSE12216. (F) KEGG analysis of these co-expressed DEGs between the GSE24129 and GSE12216. (G) PPI network of these co-expressed DEGs between the GSE24129 and GSE12216. DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction.

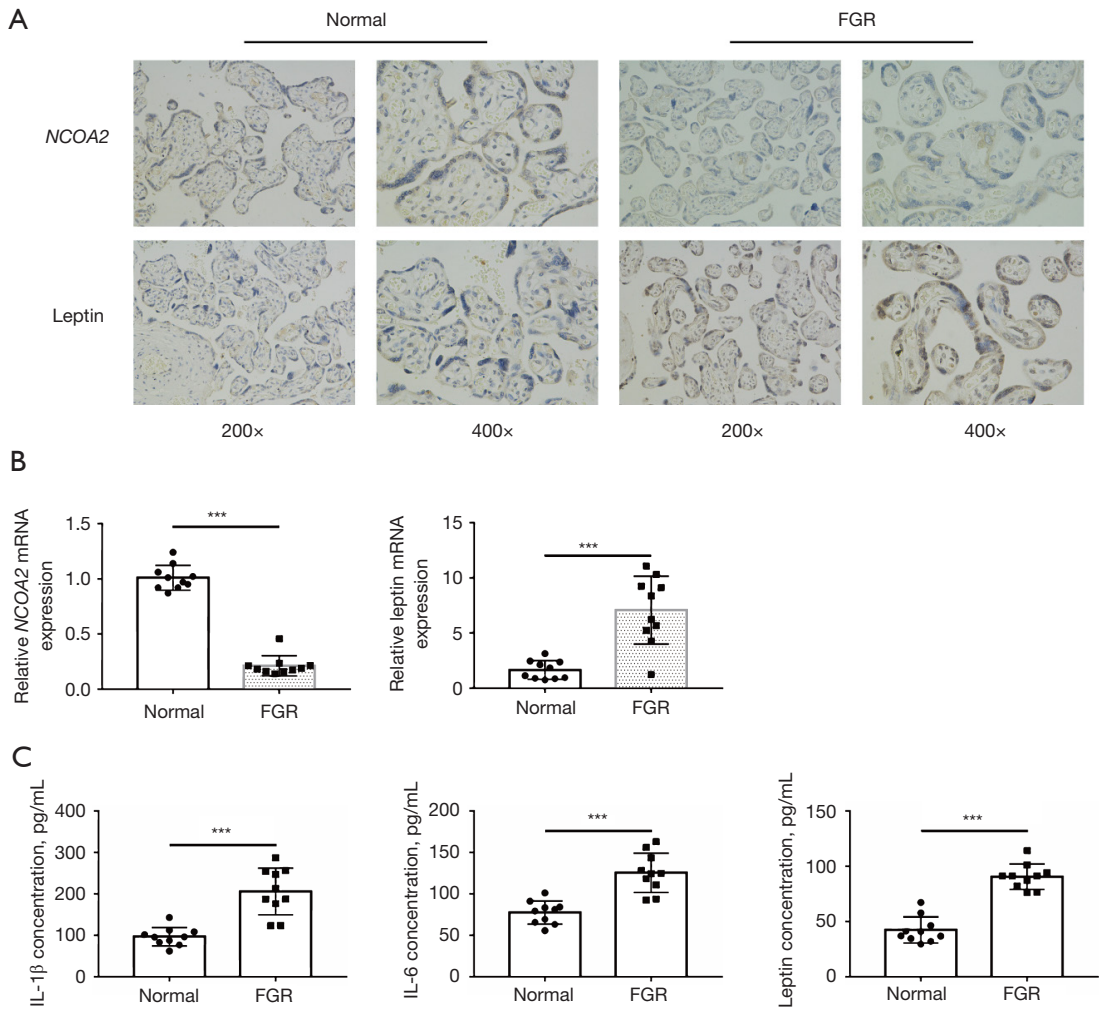


Figure 2 Validation of the *NCOA2* expression and leptin secretion in FGR tissues. (A) IHC staining of *NCOA2* and leptin in FGR patients and normal person. (B) RT-PCR assay analyzed the expression of *NCOA2* and leptin in FGR patients and normal person. (C) ELISA assay analyzed the expression of IL-1β, IL-6, and leptin in FGR patients and normal controls. Statistical evaluation was performed using one-way ANOVA. Mean ± SEM. ***, P<0.001; FGR, fetal growth restriction; IHC, immunohistochemistry; ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1β; IL-6, interleukin-6; RT-PCR, real-time quantitative polymerase chain reaction; ANOVA, analysis of variance; SEM, standard error of mean.

NCOA2 overexpression promoted proliferation, migration, and inhibited apoptosis and the secretion of leptin in trophoblast cells

LPS stimulation is an established method that induces an FGR-like phenotype in trophoblast cells (24). We further evaluated the function of *NCOA2* by overexpressing *NCOA2* in LPS-treated trophoblasts. The proliferation of *NCOA2* overexpression in LPS-stimulated HTR-8/SVneo cells was detected by CCK-8 and colony formation assay. As shown in *Figure 3A*,*3B*, LPS treatment suppressed cell proliferation whereas overexpression of *NCOA2* enhanced cell proliferation as compared with the negative control. The significance of *NCOA2* overexpression in apoptosis was detected by flow cytometry. LPS stimulation promoted both early and late apoptosis whereas overexpression of *NCOA2* inhibited both early and late apoptosis in HTR-8/SVneo cells compared with the controls (*Figure 3C*). Furthermore, the significance of *NCOA2* overexpression in cell migration was studied using Transwell assay. The overexpression of *NCOA2* group had more cells migrated to the bottom

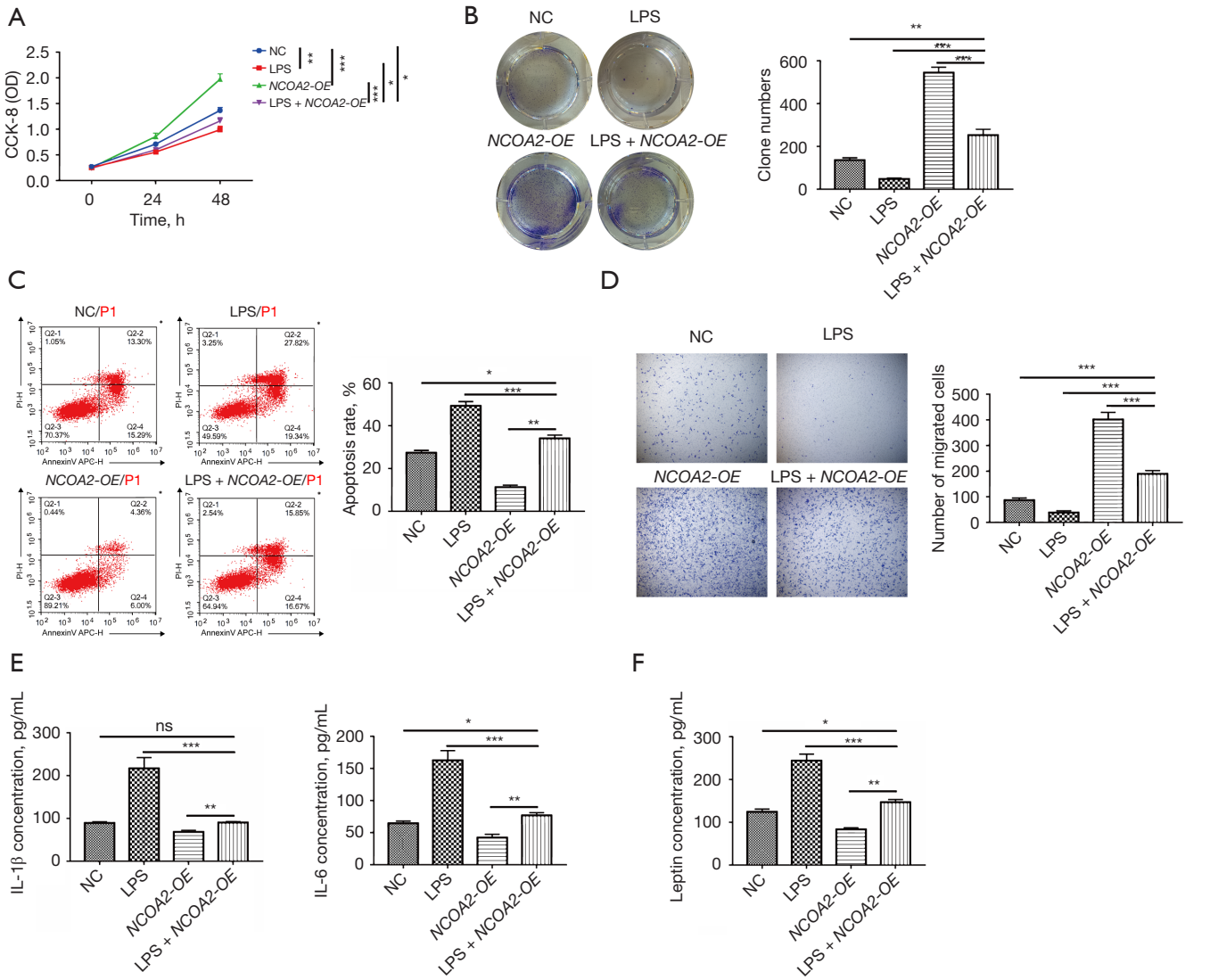


Figure 3 *NCOA2* overexpression promoted proliferation and migration, and inhibited apoptosis and the secretion of leptin in trophoblast cells. (A) CCK-8 analysis of LPS-stimulated HTR-8/SVneo cells with the treatment of *NCOA2* overexpression or not. (B) Colony formation analysis of LPS stimulated HTR-8/SVneo cells with the treatment of *NCOA2* overexpression or not. The clones were dyed crystal violet and photographed up close with a Single Lens Reflex camera. (C) Apoptosis of LPS-stimulated HTR-8/SVneo cells with the treatment of *NCOA2* overexpression or not by flow cytometry. (D) Transwell assay of LPS stimulated HTR-8/SVneo cells with the treatment of *NCOA2* overexpression or not. The migrated cells were stained with crystal violet and photographed with an inverted microscope at a 40× multiplex. (E) ELISA assay analyzed the expression of IL-1β, IL-6, and leptin (F) in LPS-stimulated HTR-8/SVneo cells with the treatment of *NCOA2* overexpression or not. Statistical evaluation was performed using one-way ANOVA. Mean ± SEM. ns, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001. *NCOA2-OE* is the same as *NCOA2* overexpression, and *NCOA2-sh* is the same as *NCOA2* knockdown. CCK-8, Cell Counting kit-8; OD, optical density; NC, negative control; LPS, lipopolysaccharide; OE, over-expression; IL-1β, interleukin-1β; IL-6, interleukin-6; ELISA, enzyme-linked immunosorbent assay; SEM, standard error of mean.

chamber, but the LPS group, by contrast, had less cells than the negative control group (*Figure 3D*).

It has recently been shown that inflammation is an important etiological factor in FGR and that LPS treatment could lead to an FGR-like phenotype (25). As shown in *Figure 3E*, the expression level of IL-1β and IL-6 was significantly increased after LPS stimulation in HTR-8/SVneo cells, whereas overexpression of *NCOA2* inhibited the secretion of IL-1β and IL-6. Moreover, the level of leptin was also suppressed in *NCOA2* overexpression (*Figure 3F*). These data indicated that *NCOA2* overexpression promoted proliferation and migration and inhibited apoptosis and the secretion of leptin in trophoblast cells.

NCOA2 aggravated functional impairment induced in HTR8/SVneo cells by LPS through the NF-κB pathway

Previous studies have shown that LPS induces the activation of NF-κB signaling pathways which lead to an inflammatory response (26-28). Recent study has uncovered the important role of the NF-κB signaling pathways in regulating inflammation during the dysfunction of trophoblast cells (29). In addition, leptin has been shown to induce direct non-specific stimulation of the IL-6 receptor, leading to NF-κB activation, and to exert anti-proliferative effects (30). In the PPI network analysis, we found a regulation interaction between *NCOA2* and leptin, we also found that *NCOA2* overexpression suppressed the secretion of leptin during LPS stimulation in HTR-8/SVneo cells. Therefore, we hypothesized that *NCOA2* participated in the process of FGR via activation of the NF-κB signaling pathway. The expression levels of IκBα, NF-κB/p65, and p-NF-κB/p65 were detected in *NCOA2* overexpressed HTR-8/SVneo cells after stimulation with LPS. The results indicated that the expression levels of IκBα were decreased and those of p-NF-κB/p65 were significantly increased in LPS-treated HTR8/SVneo cells compared with negative control (*Figure 4A*). Conversely, *NCOA2* overexpression increased the expression levels of IκBα and reduced the p-NF-κB/p65 compared to the LPS treatment group (*Figure 4A*).

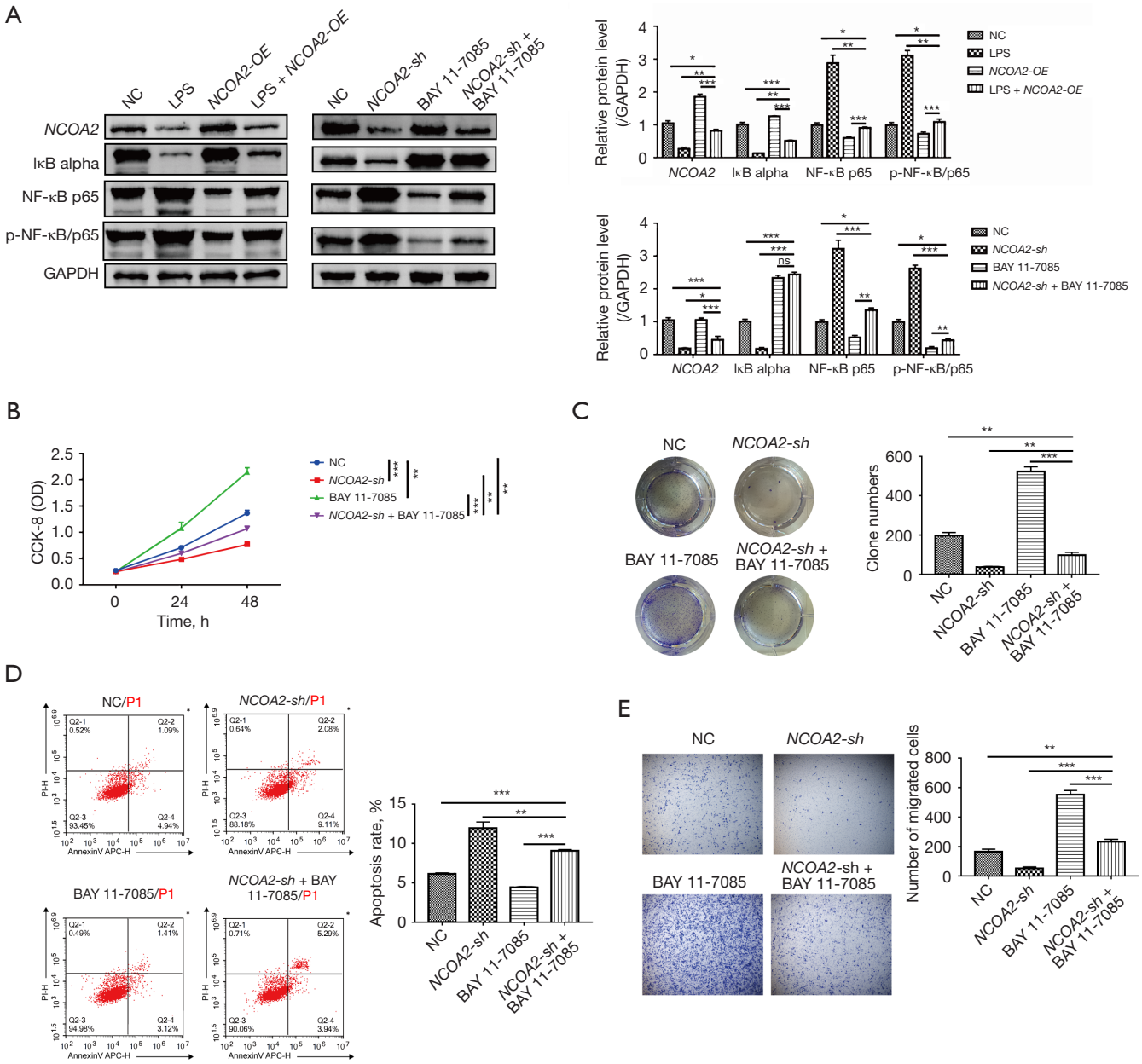
We then examined the role of NF-κB pathway in *NCOA2* knockdown HTR-8/SVneo cells function using an NF-κB pathway inhibitor, BAY 11-7085. Cell function experiments showed compared with the control group, the *NCOA2* knockdown group had reduced cell proliferation and migration, and promoted apoptosis. After adding BAY 11-7085, the *NCOA2* knockdown + BAY 11-7085 group exhibited enhanced cell proliferation and migration, and inhibited apoptosis (*Figure 4B-4E*). Moreover, the level of IL-1β, IL-6 and leptin were significantly increased (*Figure 4F,4G*) in *NCOA2* knockdown HTR-8/SVneo cells, whereas BAY 11-7085 treatment rescued the regulation by NCOA2 knockdown. The above results indicated that *NCOA2* aggravated the LPS-induced functional impairment in HTR8/SVneo cells through the NF-κB pathway.

Discussion

FGR, formerly called IUGR, refers to a condition in which an unborn baby is smaller than it should be because it is not growing at a normal rate inside the womb. A variety of perinatal adverse pregnancy outcomes as well as a variety of childhood and adult diseases are associated with the occurrence of FGR (31). FGR, a disease affected by many factors, is one of the most common and difficult diseases in obstetrics because of its poor prognosis and therapeutic effect. The placenta is the tissue that joins the mother and fetus, carrying oxygen and nutrients to the baby and permitting the release of waste products from the baby. Most cases of FGR are caused by failure of the placenta (3). FGR placental pathological features are local inflammatory response, increased apoptosis of placental trophoblastic cells, and remodeling disorders of the spiral artery (32). The role and mechanisms of placental inflammation in the pathogenesis of FGR need to be further explored.

In this study, we focused on identifying DEGs in the presence of FGR pathogenesis compared with control pregnancies from the GEO database using R software and found that *NCOA2* might be the candidate gene. This was further validated by qRT-PCR and IHC staining. The expression of *NCOA2* was significantly decreased in the placentae from FGR pregnancies compared with control cases. *NCOA2*, also known as glucocorticoid receptor-interacting protein 1 (*GRIP1*), steroid receptor coactivator-2 (*SRC-2*), or transcriptional mediators/intermediary factor 2 (*TIF2*), is a member of the p160 family of transcriptional coactivators. *NCOA2* controls multiple physiological processes, including glucose homeostasis, energy metabolism, and reproduction (33). Interestingly, *NCOA2* is widely known for its oncogenic role, including endometrial cancer, pleural cancer, and breast cancer (34). However, there is still a lack of research on the association between *NCOA2* and FGR pathogenesis.

It has been reported that abnormal maternal inflammation is often associated with the occurrence



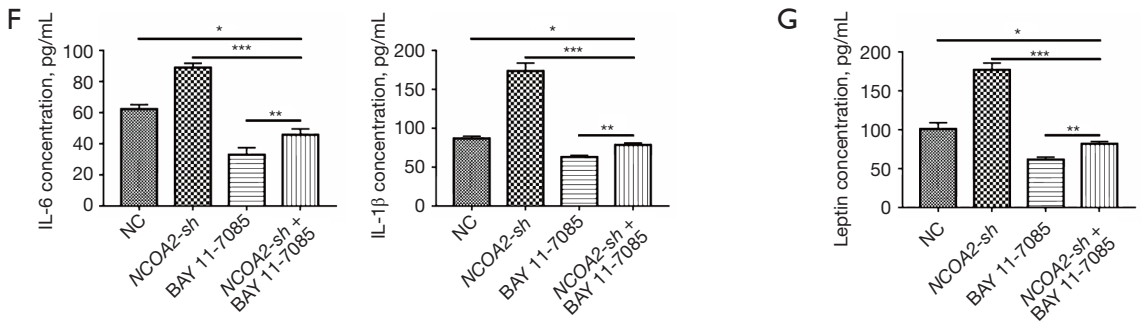


Figure 4 *NCOA2* aggravated functional impairment induced of HTR8/SVneo cells by LPS through the NF-κB pathway. (A) WB assay analyzed the expression of *NCOA2*, leptin, IκBα, NF-κB/p65 and p-NF-κB/p65 in *NCOA2* knockdown or not HTR-8/SVneo cells pretreated with NF-κB inhibitor BAY 11-7085. (B) CCK-8 analysis of *NCOA2* knockdown or not HTR-8/SVneo cells pretreated with NF-κB inhibitor BAY 11-7085. (C) Clone formation analysis of *NCOA2* knockdown or not HTR-8/SVneo cells pretreated with NF-κB inhibitor BAY 11-7085. The clones were dyed crystal violet and photographed up close with a Single Lens Reflex camera. (D) Apoptosis of *NCOA2* knockdown or not HTR-8/SVneo cells pretreated with NF-κB inhibitor BAY 11-7085 by flow cytometry. (E) Transwell assay of *NCOA2* knockdown or not HTR-8/SVneo cells pretreated with NF-κB inhibitor BAY 11-7085. The migrated cells were stained with crystal violet and photographed with an inverted microscope at a 40× multiplex. (F) ELISA assay analyzed the expression of IL-1β, IL-6, and leptin (G) in *NCOA2* knockdown or not HTR-8/SVneo cells pretreated with NF-κB inhibitor BAY 11-7085.Statistical evaluation was performed using one-way ANOVA. Mean ± SEM. ns, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001. *NCOA2-OE* is the same as *NCOA2* overexpression, and *NCOA2-sh* is the same as *NCOA2* knockdown. NC, negative control; LPS, lipopolysaccharide; OE, over-expression; CCK-8, Cell Counting Kit-8; OD, optical density; IL-6, interleukin-6; IL-1β, interleukin-1β; WB, western blot; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; SEM, standard error of mean.

of FGR. These inflammatory factors may induce the increase of trophoblast apoptosis, placental vasculogenesis, and dysfunction, which may affect the nutritional exchange between mother and fetus, and further lead to the occurrence of FGR (35). Moderate trophoblast migration and invasion ability is the key to establish, maintain and terminate physiologic pregnancy in time. When the migration and invasion ability of trophoblast cells is too weak, it can lead to abortion, premature delivery, fetal growth retardation (FGR), intrauterine fetal death, gestational hypertension and other pathologic pregnancy (36). As expected, the expression level of the proinflammatory cytokines, IL-1β and IL-6, was significantly increased in the placental tissues of FGR pregnancies compared with the control pregnancies (37). These data indicate that *NCOA2* and leptin may participate in the progress of FGR and placental inflammation was also associated with the FGR pathogenesis.

Leptin, a non-glycosylated peptide of 146 amino acid residues (16 kDa), is an adipocyte-derived hormone that acts as a major regulator for food intake and energy homeostasis. Leptin deficiency or resistance can result in profound obesity, diabetes, and infertility in humans (38). Leptin has been described as a multitasking cytokine in pregnancy, particularly in the placenta, and may have paracrine or/and autocrine functions (39). Leptin metabolism and function alterations in the placenta are linked to different disorders during pregnancy, such as gestational diabetes, IUGR, recurrent miscarriage, and preeclampsia. Leptin levels are also elevated in obese pregnant women correlating with more pronounced insulin resistance, which might contribute to fetal overgrowth (40). In this study, we found that the expression of leptin was increased in FGR patients compared with normal controls. The secretion of leptin in the serum of FGR patients was also increased, suggesting that high levels of leptin could promote the progression of FGR, providing a potent marker for FGR screening.

FGR is characterized by impaired trophoblast function and LPS stimulation is an established method that induces FGR like phenotype in trophoblast cells. In this study, we report that LPS treatment inhibited the function of trophoblast cells, whereas overexpression of *NCOA2* promoted the proliferation and migration, and inhibited the apoptosis and the secretion of proinflammatory cytokines such as IL-1β and IL-6 of LPS-induced trophoblast cells. The NF-κB signaling pathway may contribute

to the pathology of FGR and play key roles in many cellular processes, such as cell metabolism, inflammatory processes, proliferation, migration, and apoptosis. Transcription factors may recruit transcriptional activators and provide access to the regulatory sequences located on gene promoters to allow gene transcription activation or repression. Zhou *et al.* found that NF-κB formed a complex with *NCOA2* in response to LPS and then selectively transactivated the expression of proinflammatory cytokines (41). Therefore, we hypothesized that *NCOA2* participates in the process of FGR via activation of the NF-κB signaling pathway. Based on the results of our western blot assays, the protein expression of IκBα was increased whereas the p-NF-κB/p65 ratio was decreased in *NCOA2*-overexpressed LPS-stimulated trophoblast cells. Functional experiments with the NF-κB signaling pathway inhibitor BAY 11-7085 also demonstrated that *NCOA2* may function through targeting the NF-κB pathway and eventually lead to FGR.

Conclusions

In summary, the present study highlights the correlation between *NCOA2* and FGR. *NCOA2* overexpression promoted proliferation and migration, and inhibited apoptosis, in LPS-stimulated trophoblast cells via the NF-κB pathway. In addition, our data also demonstrated that the level of leptin in serum was associated with the progression of FGR. Therefore, this study provides a basis for the pathogenesis of FGR and a promising potential target for treatment.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-6444/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-6444/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6444/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Research Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (No. 2020-SR-434). All participants signed a written informed consent.

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